IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Examiner	:	Fox, David
Art Unit	:	1638
Applicants	:	Henry Daniell
Serial No.	:	09/079,640
Docket No.	:	10742-002
Filed	:	May 15, 1998
For	:	Universal Chloroplast Integration and Expression Vector, Method of Use and Transformed Plants

DECLARATION OF HENRY DANIELL, PhD.

I, Henry Daniell, Ph.D. hereby declare and say as follows:

THAT, I am employed as Pegasus Professor & Trustee Chair at University of Central Florida, Orlando, FL.;

THAT, I am the above-named Applicant and inventor of the subject matter described and claimed in the above-identified patent application;

THAT, by virtue of my educational and employment background, my leadership at national/international scientific conferences, my ongoing research, my continuing review of scientific literature, and through correspondence with professional colleagues, I am aware of the level of skill of one ordinarily skilled in the art of plant genetics, and in particular, chloroplast transformation;

THAT, I have studied the application Serial No. 09/079,640 and office actions which have been issued during prosecution of this application, as well as responses which have been filed on the Applicants' behalf, and being thus duly qualified declare as follows:

1. The Patent Office has rejected pending claims 3, 171, 190-191, 193, 196, 214 and 216-223, as lacking enablement. I also understand that this Declaration is being filed in conjunction with a response that amends claims 190-191, *inter alia*. The statements made herein relate to the question of enablement of the aforementioned rejected claims.

2. As a foundation for discussing the rejection specified in paragraph 1, as well as other rejections, it should be helpful to clarify the terms "transcriptionally silent", "read-through" and "transcriptionally active", as used in the context of chloroplast genome spacer regions. Attention is drawn to Exhibit A , which is a diagram depicting the differences between transcriptionally active, transcriptionally silent and read-through regions in chloroplast genomes. The term "transcriptionally silent" pertains to a region located between two known divergent promoters of chloroplast genes located on opposite strands where the promoters transcribe these genes in opposite directions away from the silent region of the genome. See pages 5-6 of the application. The term "transcriptionally active" pertains to a region located between an upstream promoter and a downstream terminator. Such locations are often present in operon regions, or polycistronic transcription units, where several genes are co-transcribed by an upstream promoter. Such regions are described in the Sugita et al. (1996) reference, which is of record and has been the subject of past correspondence with the Patent Office. The term "read-through" pertains to a region that is located downstream from a terminator and upstream from a promoter. It is a known phenomenon that chloroplast terminators can be inefficient at terminating transcription. As a result, DNA introduced at a read-through region can sometimes be transcribed due to spillover of the transcript past the terminator, but such transcription is unpredictable.

3. There are a number of known transcriptionally active spacer regions in the chloroplast genome. Sixty (60) operons or polycistronic transcription units were known at the time of the filing of the present application, as was taught in the Sugita et al. (1996) reference. At the time of filing the present application, the conventional wisdom was that only regions between opposing promoters, i.e., the now defined transcriptionally

silent regions, were appropriate for introducing foreign genes. This point has been established during the course of prosecution of the present application. However, contrary to such conventional wisdom, I persevered to determine whether one might be able to introduce and express foreign genes into regions of the chloroplast genome that were not transcriptionally silent. I discovered that the now defined "transcriptionally active" sites could successfully and reproducibly be implemented for chloroplast transformation. The results of my initial studies are provided in the present application. Since this initial work, my lab and other labs have successfully demonstrated introduction and expression of several different genetic constructs. In disproving well-accepted dogma about what regions were operable for chloroplast transformation, those skilled in the art, now equipped with the teachings provided in the present application coupled with the knowledge of those skilled in the art at the time (particularly the information provided by the Sugita et al. reference) could now test other known operon or polycistronic transcription units within the same species or genome for introduction and expression of foreign DNA. This could be achieved with straight-forward molecular biology techniques well-known at the time of filing and still in use today. Though testing "transcriptionally active" regions other than the intergenic spacer 2 region might require some experimentation, by no means would such testing of other regions be uniquely difficult or cumbersome when performed within the same species or genome. In other words, testing other transcriptionally active regions is achievable through routine experimentation and methods and would not require undue experimentation.

4. The attached Table 1 (Exhibit B) sets forth several different studies that to my knowledge demonstrate the successful chloroplast transformation utilizing the intergenic spacer 2 region, which were conducted after filing the present application. The attached Table 2 (Exhibit C) sets forth several different studies conducted after the filing of the present application that to my knowledge demonstrate successful chloroplast transformation utilizing transcriptionally silent and read-through regions. Provided in paragraph 2 is a definition of transcriptionally active, transcriptionally silent and read-through regions. Though such regions have structural differences, the same processes and mechanisms of transcription apply equally to each within the

same species or genome. From a scientific perspective, there is nothing uniquely difficult about testing transcriptionally active regions in comparison to transcriptionally silent or read-through regions. The successful testing and implementation of numerous different transcriptionally silent and read-through regions for chloroplast transformation in fact serves as evidence that transcriptionally active spacer regions other than the intergenic spacer 2 region may also be tested and successfully implemented within the same species or genome. In my lab, there has not been a motivation to test and characterize other transcriptionally active regions because the intergenic spacer 2 regions have been successfully utilized for different chloroplast transformation studies using different constructs. However, I have no doubt that other regions could be tested and implemented with routine experimentation.

5. The undersigned declares further that all statements made herein are of his own knowledge are true and that all statements made on information in belief are believed to be true and that such willful false statements made jeopardize the validity of the application or of any patent issuing thereon.

Further declarant sayeth naught.

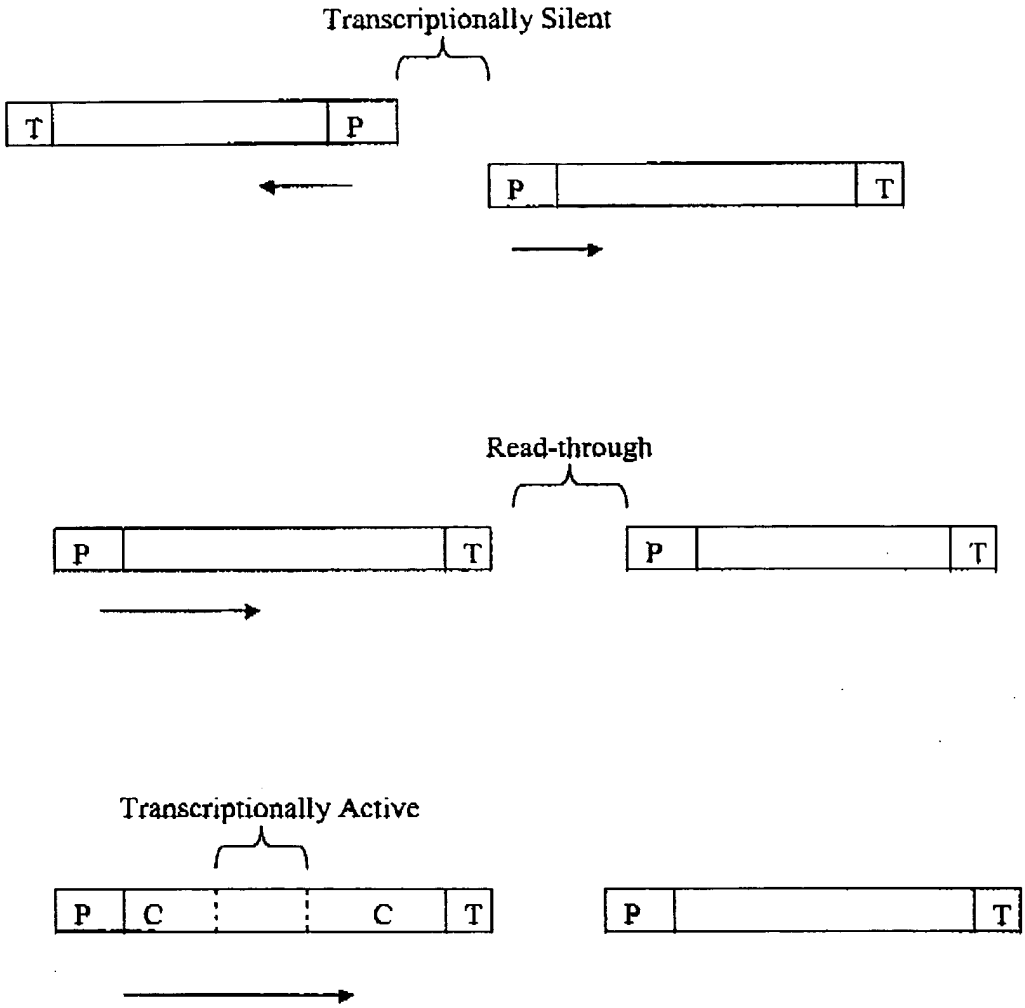
H Daniell

Henry Daniell, Ph.D.

July 29, 2005

Date

EXHIBIT A



P = promoter
C = cistron
T = terminator

Arrows → represent direction of transcription

EXHIBIT B

Table 1: List of genes integrated into the *trn*I-*trn*A intergenic spacer region of the rRNA operon within the chloroplast genome.

INSERTION SITE	TRANSCRIPTION STATUS	TRANSGENE INTEGRATED	% TOTAL SOLUBLE PROTEIN	Protein Size (kDa)	REF
*trn*I/*trn*A	Active (same strand, promoterless spacer region)	*aadA*/*aroA*^{D}	ND	47.623	3
		aadA/*ctxB*^{D}	4%	11.6	9
		aadA/*ltxB*^{D}	2.5%	11.6	43
		aadA/*ctxB*-CPV^{D}	31.1%	14.0	11
		aadA/gfp-CPV^{D}	22.6 %	29.0	11
		aadA/*pag*^{D}	18.1 %	83.0	10
		aadA/CaF1-Lcrv^{D}	14.8%	53.0	19, 44
		aadA/EG121^{D}	ND		31
		aadA/MSI-99^{D}	21.5%	2.381	5
		aadA/IGF-1^{D}	33%	7.6	18, 45
		aadA/INFa5^{D}	ND	23.0	18, 46
		aadA/INF-a2b^{D}	19%	21.5	18, 47
		aadA/HSA^{D}	11.1%	66.5	17
		aadA/Guy's 13^{D}	ND	50.5, 23.6	18
		aadA/*Cry*2Aa2^{D}	2-3%	65.0	4
		aadA/*Cry*2Aa2 operon^{P}	46.1%	65.0	20
		aadA/*tps*^{D}	ND	56.0	6
		aadA/*mer*A-merB^{P}	ND	69.0, 24.0	8
		aadA/*badh*^{D} (*Daucus carota*, Carrot)	ND	54.275	13 7
		aadA/*Rbc*S^{D,M}	ND	14.559	41
		aadA^{M}	ND	29.447	48
		nptII/*aphA6*^{D,M} *Gossipium hirsutum*, Cotton	ND	ND	24
		aadA/gfp^{D}	ND	62.0	49
		aadA/*ubiC*^{M}	35%	ND	54
		D – Dicistron; **P** – Polycistron; **M** – Monocistron			

EXHIBIT C

Table 2: List of other integration sites used for chloroplast transformation and their transcriptional status.

Insertion Site	Transcription Status	Ref
trnH/psbA	Read-through, Same strand with promoter	50
*trn*G/*trn*fM (*L. esculentum*, *tomato*)	Silent, divergent genes, opposite strands	51 23
*ycf*3/*trn*S	Silent, divergent genes, opposite strands	52, 42
*rbc*L/*acc*D (*S. tuberosum*, potato)	Read-through, Same strand with promoter	53, 3 22
*pet*A/*psb*J	Silent, divergent genes, opposite strands	42, 52
*pet*D/*rpo*A	Silent, divergent genes, opposite strands	42
3_*rps*12/*trn*V (*A. thaliana*) (*Glycine max*)	Silent, divergent genes, opposite strands	56 33 25
*trn*V/*rrn*16 (*S. tuberosum*)	Read-through, Same strand with promoter	57 22
*trn*N/*trn*R	Silent, divergent genes, opposite strands	52, 58
*rpl*32/*trn*L	Read-through, Same strand with promoter	59, 60, 61

References

3. Daniell, H., Datta, R., Varma, S., Gray, S. and Lee, S.B. (1998) Containment of herbicide resistance through genetic engineering of the chloroplast genome. *Nat. Biotechnol.* **16**, 345-348.

4. Kota, M., Daniell, H., Varma, S., Garczynski, S.F., Gould, F. and William, M.J. (1999) Overexpression of the *Bacillus thuringiensis* (Bt) Cry2Aa2 protein in chloroplasts confers resistance to plants against susceptible and *Bt*-resistant insects. *Proc. Natl. Acad. Sci. USA* **96**, 1840-1845.

5. DeGray, G., Kanniah, R., Franzine, S., John, S. and Daniell, H. (2001) Expression of an antimicrobial peptide via the chloroplast genome to control phytopathogenic bacteria and fungi. *Plant Physiol.* **127**, 852-862.

6. Lee, S.B., Kwon, H.B., Kwon S.J., Park S.C., Jeong M.J., Han S.E., Byun M.O. and Daniell, H. (2003) Accumulation of trehalose within transgenic chloroplasts confers drought tolerance. *Mol. Breeding* 11,1-13.

7. Kumar, S., Dhingra, A. and Daniell, H. (2004) Plastid expressed betaine aldehyde dehydrogenase gene in carrot cultured cells, roots and leaves confers enhanced salt tolerance. *Plant Physiol.* **136**: 2843-2854.

8. Ruiz, O.N., Hussein, H., Terry, N., Daniell, H. (2003) Phytoremediation of organomercurial compounds via chloroplast genetic engineering. *Plant Physiol.* **132**: 1-9.1344-1352.

9. Daniell, H., Lee, S.B., Panchal, T. & Wiebe, P.O. (2001) Expression of the native cholera toxin B subunit gene and assembly as functional oligomers in transgenic tobacco chloroplasts. *J. Mol. Biol.* **311**, 1001-1009.

10. Watson, J., Koya V., Leppla S. and Daniell, H. (2004) Expression of *Bacillus anthracis* protective antigen in transgenic chloroplasts of tobacco, a non-food/feed crop. *Vaccine* **22**, 4374-4384.

11. Molina, A., Hervás-Stubbs, S., Daniell, H., Mingo-Castel, A., and Veramendi J. (2004) High-yield expression of a viral peptide animal vaccine in transgenic tobacco chloroplasts. *Plant Biotech. J.* **2**, 141-153.

13. Daniell, H., Muthukumar, B. and Lee, S. B. (2001) Marker tree transgenic plants: engineering the chloroplast genome without the use of antibiotic selection. *Curr. Genet.* **39**, 109-116.

17. Fernandez-San Millan, A., Mingo-Castel, A. and Daniell, H. (2003) A chloroplast transgenic approach to hyper-express and purify human serum albumin, a protein highly susceptible to proteolytic degradation. *Plant Biotechnol. J.* 1,71-79.

18. Daniell, H., Carmona-Sanchez, O., and Burns, B. B. (2004) Chloroplast derived antibodies, biopharmaceuticals and edible vaccines. In R. Fischer & S. Schillberg (Eds.) *Molecular Farming* pp.113-133. Weinheim: WILEY-VCH Verlag.

19. Chebolu, S. and Daniell, H. (2004) Chloroplast derived vaccine antigens and biopharmaceuticals: expression, folding, assembly and functionality. *Curr. Trends Microbiol. Immunol.*, in press.

20. DeCosa, B., Moar, W., Lee, S.B., Miller, M. and Daniell, H. (2001) Overexpression of the *Bt* Cry2Aa2 operon in chloroplasts leads to formation of insecticidal crystals. *Nat. Biotechnol.* **19**,71-74

22. Sidorov, V.A., Kasten, D., Pang, S.Z., Hajdukiewicz, P.T.J., Staub, J.M., Nehra, N.S. (1999) Stable chloroplast transformation in potato: use of green fluorescent protein as a plastid marker. *Plant J.* **19**, 209-216.

23. Ruf, S., Hermann, M., Berger, I. J., Carrer, H. and Bock, R. (2001) Stable genetic transformation of tomato plastids and expression of a foreign protein in fruit. *Nat. Biotechnol.* **19,** 870-875.

24. Kumar, S., Dhingra, A. and Daniell, H. (2004) Stable transformation of the cotton plastid genome and maternal inheritance of transgenes. *Plant Mol. Biol.***56**, 203-216.

25. Dufourmantel, N., Pelissier, B., Garçon, F., Peltier, J.M. and Tissot. G. (2004). Generation of fertile transplastomic soybean. Plant Mol Biol. **55**: 727-741.

31. Guda, C., Lee, S. B. and Daniell, H. (2000) Stable expression of biodegradable protein based polymer in tobacco chloroplasts. *Plant Cell Rep.* **19**, 257-262.

32. Daniell, H., Cohill, P., Kumar, S. Dufourmantel, N. (2004) Chloroplast genetic engineering. In *Molecular biology and biotechnology of plant organelles* (Daniell, H. and Chase, C., eds), pp. 423-468. Kluwer Academic Publishers

33. Sikdar, S. R., Serino, G., Chaudhuri, S. and Maliga, P. (1998) Plastid transformation in *Arabidopsis* . *Plant Cell Rep.* **18,** 20-24.

41. Dhingra, A., Portis Jr., A. R. and Daniell, H. (2004) Enhanced translation of a chloroplast expressed *Rbc*S gene restores SSU levels and photosynthesis in nuclear antisense *Rbc*S plants. *Proc. Natl. Acad. Sci. U SA.***101**, 6315-6320.

42. Klaus, S. M. J., Huang, F. C., Eibl, C., Koop, H. U. and Golds, T. J. (2003) Rapid and proven production of transplastomic tobacco plants by restoration of pigmentation and photosynthesis. *Plant J.* **35**, 811-821.

43. Kang, T. J., Loc, N. H., Mi-Ok, J., Jang, Y. S., Kim, Y. S., Seo, J. E. and Yang, M. S. (2003) Expression of the B-subunit of E. coli heat-labile enterotoxin in the chloroplasts of plants and its characterization. *Transgenic Res.* **12**, 683-691.

44. Singleton, M.L. (2003) Expression of CaF1 and LcrV as a fusion protein for a vaccine against *Yersinia pestis* via chloroplast genetic engineering. MS thesis, University of Central Florida, USA.

45. Ruiz, G. (2002) Optimization of codon composition and regulatory elements for expression of the human IGF-1 in transgenic chloroplasts. MS thesis, University of Central Florida, USA.

46. Torres, M. (2002) Expression of interferon α5 in transgenic chloroplasts of tobacco. MS thesis, University of Central Florida, USA.

47. Falconer, R. (2002) Expression of interferon α2b in transgenic chloroplasts of a low-nicotine tobacco. MS thesis, University of Central Florida, USA.

48. Muhlbauer, S. K., Lossl, A., Tzekova, L., Zou, Z. R. and Koop, H. U. (2002) Functional analysis of plastid DNA replication origins in tobacco by targeted inactivation. *Plant J.* **32**, 175-184.

49. Jeong, S. W., Jeong, W. J., Woo, J. W., Choi, D. W., Park, Y. I. and Liu, J. R. (2004) Dicistronic expression of the green fluorescent protein and antibiotic resistance genes in the plastid for selection and tracking of plastid-transformed cells in tobacco. *Plant Cell Rep.* **22**, 747-751.

50. Carrer, H. and Maliga, P. (1995) Targeted insertion of foreign genes into the tobacco plastid genome without physical linkage to the selectable marker gene. *Biotechnol.* **13**, 791-794.

51. Bock, R. and Maliga, P. (1995) Correct splicing of a group II intron from a chimeric reporter gene transcript in tobacco plastids. *Nuc. Acids Res.* **23**, 2544-2547.

52. Huang, F. C., Klaus, S. M. J., Herz, S., Zou, Z., Koop, H. U., Golds, T. J. (2002) Efficient plastid transformation in tobacco using the *aph*A-6 gene and kanamycin selection. *Mol. Genet. Genomics* **268**, 19-27.

53. Svab, Z. and Maliga, P. (1993) High frequency plastid transformation in tobacco by selection for a chimeric aadA gene. Proc. Natl. Acad. Sci. USA 90: 913-917.

54. Viitanen, P. V., Devine, A. L., Khan, M. S., Deuel, D. L., Dyk, D. E. V. & Daniell, H. (2005). Metabolic engineering of the chloroplast genome using the *E. coli ubiC* gene reveals that chorismate is a readily abundant plant precursor for p-Hydroxybenzoic acid biosynthesis. *Plant Physiol.* in press.

56. Zoubenko, O. V., Allison, L. A., Svab, Z. and Maliga, P. (1994) Efficient targeting of foreign genes into the tobacco plastid genome. *Nuc. Acids Res.* **22**, 3819-3824.

57. Staub, J. M. and Maliga, P. (1993) Accumulation of D1 Polypeptide in tobacco plastids is regulated via the untranslated region of the *psbA* messenger RNA. *EMBO J.* **12**, 601-606.

58. Zou, Z., Eibl, C. and Koop, H. U. (2003) The stem-loop structure of the tobacco *psb*A 5' UTR is an important determinant of mRNA stability and translation efficiency. Mol. Gen. Genom. **269**, 340-349.

59. Eibl, C., Zou, Z. R., Beck, A., Kim, M., Mullet, J. and Koop, H. U. (1999) *In vivo* analysis of plastid *psb*A, *rbc*L, *rpl*32 UTR elements by chloroplast transformation: tobacco plastid gene expression is controlled by modulation of transcript levels and translation efficiency. *Plant J.* **19**, 333-345.

60. Koop, H. U., Steinmuller, K., Wagner, H., Rossler, C., Eibl, C. and Sacher, L. (1996) Integration of foreign sequences into the tobacco plastome via PEG mediated protoplast transformation. *Planta* **199**, 193-201.

61. Thum, K. E., Kim, M., Morishige, D. T., Eibl, C., Koop, H. U. and Mullet, J. E. (2001) Analysis of the barley chloroplast *psb*D light responsive promoter elements in transplastomic tobacco. *Plant Mol. Biol.* **47**, 353-366.